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Microbial dehalogenation

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Novel dehalogenases have been identified recently in various bacteria that utilise halogenated substrates. X-ray studies and sequence analysis have revealed insight into the molecular mechanisms of hydrolytic dehalogenases. Furthermore, genetic and biochemical studies have indicated that reductive dehalogenases are extra-cytoplasmic corrinoid-containing iron–sulphur proteins. Sequence analysis and mutagenesis studies indicate that several dehalogenases are homologous to enzymes that carry out transformations on non-halogenated substrates.

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Introduction

Halogenated compounds are an extremely important and diverse class of environmental chemicals. Microbiological research on the biodegradation of halogenated compounds has mainly focused on the physiological processes responsible for their mineralisation and on the enzymes involved in cleavage of the carbon–halogen bond. The characterisation of dehalogenating organisms is important for their industrial application, because their physiological properties and substrate range will determine the process conditions that should be used and the range of transformations that can be obtained in practical treatment systems.

In general, degradation can be based on one of four processes: use of a halogenated compound as a carbon source and oxidisable substrate with either oxygen or nitrate as an electron acceptor; fermentative metabolism, in which a dehalogenated intermediate serves as electron acceptor; the use of a halogenated compound as an electron acceptor with liberation of halide; and co-metabolic transformation and halide release linked to any metabolic process. The last process arises through the lack of specificity of an enzyme with another physiological function.

The key reaction during microbial degradation of halogenated compounds is the actual dehalogenation. During this step, the halogen substituent, which is usually responsible for the toxic and xenobiotic character of the compound, is replaced — often by a hydrogen or a hydroxyl group. Halogen removal reduces both recalcitrance to biodegradation and the risk of forming toxic intermediates during subsequent metabolic steps. The latter may occur during oxidative conversion where the presence of halogen substituents may lead to the production of acylhalides or 2-haloaldehydes, which are reactive products owing to their electrophilicity and may cause cellular damage.

In this review, we discuss important recent findings in the field of microbial dehalogenation. Much work has been carried out on the identification and characterisation of dehalogenating enzymes, both from aerobic and anaerobic organisms (Figure 1). Several biochemical and biophysical studies on new dehalogenases have been performed, and this has led to considerable insight into their evolution, diversity and catalytic mechanisms.

Aerobic growth on halogenated aliphatic compounds

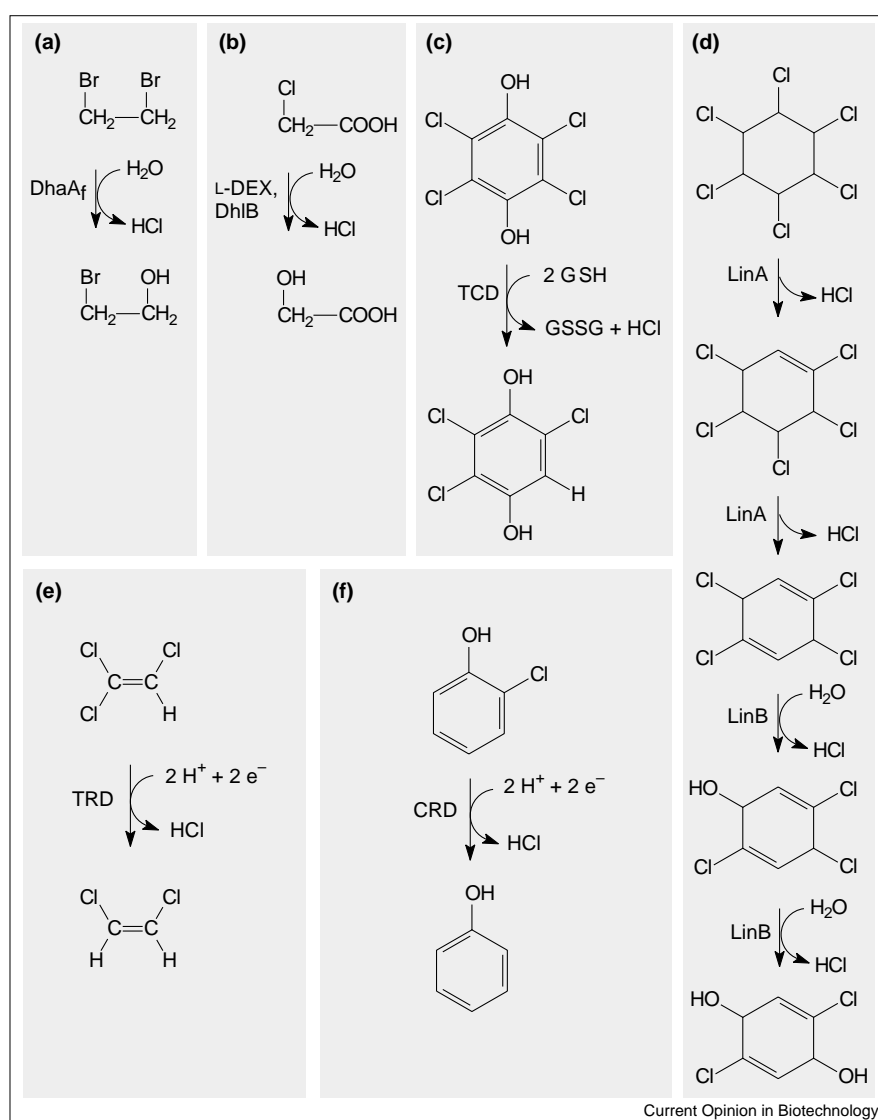
Many different organisms have been found that are capable of using halogenated compounds as a growth substrate. Relatively new examples of compounds that are known to be degradable by pure bacterial cultures include 1,3-dichloropropylene and 1,2-dibromoethane. In both cases, initial dehalogenation starts with a reaction catalysed by a hydrolytic haloalkane dehalogenase [1,2]. The haloalkane dehalogenases that degrade these compounds are homologous to the classical enzyme from *Xanthobacter autotrophicus* [3]. Even though sequence similarities are low, the overall structures are very similar and the catalytic residues are conserved [4]. Another example of a recently studied hydrolytic dehalogenase is LinB from *Sphingomonas paucimobilis*; this enzyme is involved in the conversion of tetrachlorocyclohexadiene, an intermediate in the γ -hexachlorocyclohexane degradation pathway (Figure 1) [5].

All these hydrolytic dehalogenases belong to the α/β -hydrolyase fold superfamily and comprise a main domain and a cap domain. The active site is located between these two domains and catalysis proceeds through a covalent enzyme–substrate intermediate. The enzymes possess a catalytic triad formed by residues of the main domain, with an aspartate as the nucleophile that displaces the halogen from the substrate. In addition, these enzymes have a conserved tryptophan residue in the main domain and variable residues (phenylalanine or asparagine) in the cap domain that contribute to halide binding [4,5].

Other hydrolytic dehalogenases that have recently been studied in more detail are the haloacid dehalogenases, which can be divided into at least two groups, known as group I and II, according to Hill *et al.* [6•]. Like the haloalkane dehalogenases, the group II enzymes use covalent catalysis, and detailed insight into the reaction mechanism has been obtained. X-ray structures have been solved both for chloropropionic acid dehalogenase (L-DEX) of *Pseudomonas* sp. YL [7•] and chloroacetate dehalogenase (DhlB) of *X. autotrophicus* GJ10 [8•]. The group II enzymes usually convert L-2-chloropropionic acid, but not D-2-chloropropionic acid, with an inversion of configuration at the chiral centre.

Figure 1

Recently explored dehalogenation reactions in bacterial cultures. (a) Dehalogenation of 1,2-dibromoethane by a haloalkane dehalogenase (DhaA_f) [2]. (b) Dechlorination of chloroacetate by a group II dehalogenase (L-DEX or DhlB) [6•,7•,8]. (c) Reduction of tetrachlorohydroquinone during pentachlorophenol metabolism by tetrachlorohydroquinone dehalogenase (TCD) [12]. (d) Initial dehalogenation steps during the metabolism of lindane. Two dehydrohalogenations are followed by two hydrolytic steps [5,22,23•]. (e) Reduction of trichloroethene by a reductive dehalogenase (TRD) [15••]. (f) The activity of *ortho*-chlorophenol reductive dehalogenase (CRD) [17,18].



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The group II dehalogenases have a structure that is very different from that of haloalkane dehalogenases; however, they exhibit similarity to many phosphatases, the catalytic subunits of P-type ATPases, and several other enzymes of the haloacid dehalogenase superfamily. Group II dehalogenases share a conserved nucleophilic aspartate residue that is located close to the N terminus and is involved in formation of the covalent intermediate. The aspartate is positioned by interaction with a conserved lysine. An arginine, an asparagine and a phenylalanine residue are also conserved and are involved in binding of the halogen/halide. Thus, both the nucleophilic site and the leaving group site are different from those in α/β -hydrolase fold enzymes.

Although no structural information is available as yet, biochemical studies have indicated that group I haloacid dehalogenases [8•,9], which include a remarkable enzyme

selective for D-2-chloropropionic acid and an enzyme that converts trichloroacetate to carbon monoxide [10], do not use a covalent mechanism for catalysis. In multiple turnover experiments using ¹⁸O-labelled water and the DL-DEX haloacid dehalogenase from *Pseudomonas* sp. 113, incorporation of ¹⁸O into the enzyme was not detected. Thus, Nardi-Dei *et al.* [9] proposed that the dehalogenase directly activates a water molecule to attack the α carbon of 2-haloalkanoic acid, thereby displacing the halogen atom. It has not yet been established which amino acids are involved in the dehalogenation, although a number of candidates have been highlighted by mutagenesis experiments.

Aerobic growth on halogenated aromatics

The dehalogenation of aromatic substrates may occur before, during or after cleavage of an aromatic ring. Recently, genetic and biochemical studies have yielded new insight into these mechanisms.

Cleavage of carbon–chlorine bonds before ring cleavage seems to be an important reaction in the metabolism of polyhalogenated compounds such as tetrachlorobenzene. This reaction, catalysed by a dioxygenase, was first implicated in the dehalogenation of fluorocatechol, but its scope appears to be much broader. For example, the dioxygenase TecA of *Burkholderia* PS12, which has a Rieske-type 2Fe–2S cluster, can incorporate two oxygen atoms in 1,2,4,5-tetrachlorobenzene; this is accompanied by dehydrodehalogenation, meaning that a catechol is formed immediately without the involvement of a dehydrogenase, which would be needed for a dihydrodiol to be produced. The α subunit (TecA1) seems to possess the essential determinants for dehalogenation, and more specifically Beil *et al.* [11] have proposed that a non-bulky amino acid at position 220 is required for interaction with tetrachlorobenzene and dioxygenation.

A second mechanism for dehalogenation before ring cleavage is used by tetrachlorohydroquinone reductive dehalogenase (TCD) — an enzyme involved in pentachlorophenol metabolism in *Sphingomonas chlorophenolica* (Figure 1). This enzyme catalyses the reductive displacement of a chlorine from a ring structure by a glutathione-dependent reaction; the enzyme has sequence similarity to glutathione transferases.

Anandarajah *et al.* [12] proposed recently that this dehalogenase has evolved from a maleylacetoacetate isomerase — an enzyme that isomerises a *cis* double bond to a *trans* double bond. Such an isomerase is involved in tyrosine metabolism, and it appeared that the isomerase from eukaryotic organisms shares significant sequence similarity with the reductive dehalogenases of *S. chlorophenolica* and *S. paucimobilis* that are involved in the degradation of chlorophenols and lindane (γ -hexachlorocyclohexane), respectively.

A third mechanism for dehalogenation before ring cleavage is used by 4-chlorobenzoyl coenzyme A (CoA) dehalogenase. In this case, cleavage of the carbon–halogen bond is hydrolytic; it requires the growth substrate 4-chlorobenzoate to be activated to a CoA derivative, a process that is found in *Pseudomonas* CBS3. The dehalogenase is evolutionarily related to a 2-enoyl-CoA hydratase [13].

Dechlorination during ring cleavage of catechol has been assumed to be a non-realistic option for a long time. In fact, it has been proposed that *meta* cleavage of an aromatic ring between a hydroxy- and a halogen-substituted carbon atom is prohibited because it would yield an unstable and toxic acyl halide. Surprisingly, a *meta* cleavage enzyme, 3-chlorocatechol dioxygenase from the chlorobenzene-degrading organism *Pseudomonas putida* GJ31, rapidly cleaves and dechlorinates 3-chlorocatechol to produce 2-hydroxymuconic acid. Our studies with hybrid enzymes have shown that this activity is linked to the C-terminal part of the ring-cleavage dioxygenase [14••]. Thus, this enzyme is resistant to inactivation by potential reactive intermediates or prevents their formation by efficient dechlorination. It remains

to be established how widely distributed dehalogenation by *meta* cleavage is.

Anaerobic growth on halogenated substrates: halorespiration

In halorespiration, a halogenated compound serves as a terminal electron acceptor during the oxidation of an electron-rich compound, such as hydrogen or an organic substrate. The biochemistry of anaerobic reductive dehalogenation is diverse. Three different reductive dehalogenases have been identified and their genes have been cloned and sequenced. These are the trichloroethene reductive dehalogenase from *Dehalococcoides ethenogenes* (Figure 1) [15••], tetrachloroethene reductive dehalogenase from *Dehalospirillum multivorans* [16] and *ortho*-chlorophenol reductive dehalogenase from *Desulfitobacterium dehalogenans* [17,18].

The trichloroethene reductive dehalogenase from *D. ethenogenes* dehalogenates trichloroethene to ethene in three consecutive steps, and is also active with 1,2-dichloroethane and 1,2-dibromoethane. *D. ethenogenes* can completely dechlorinate perchloroethene to ethene, using an initial reductive dehalogenase for conversion of tetrachloroethene or trichloroethene, and another reductive enzyme for ethene formation. Genome sequencing, however, has shown that several more reductive dehalogenases may be present in *D. ethenogenes*. Remarkably, the only known functional electron acceptors are chlorinated compounds, which raises questions about the evolutionary origin of *D. ethenogenes* and its natural ecological niche [19]. The presence of a twin arginine signal sequence suggests that the reductive dehalogenases are extra-cytoplasmic enzymes. Electrons should thus flow to electron-transport components that generate a proton motive force for ATP synthesis.

Reductive dehalogenases have a molecular weight of 50–65 kDa, and contain a cobalamin cofactor and iron–sulphur clusters. Sequence similarity to other proteins is confined to the C-terminal part of the protein, which has two binding motifs for [4Fe–4S] clusters, each consisting of four cysteines. In addition, genetic analysis has shown that there is a small protein encoded by a gene closely linked to the dehalogenase gene; it has been proposed that this small protein is involved in either membrane association or electron transfer from the dehalogenase to membrane components [15••,17].

Regulation of dehalogenase gene expression

A dechlorination reaction often requires only a single protein that can recognise and convert a xenobiotic substrate; however, regulated expression by means of binding of a halogenated substrate and interaction with the transcription machinery requires a second protein. Therefore, if the synthesis of a dehalogenating enzyme is subject to regulation, the pathway must be more evolved than in the case of constitutive protein expression [2].

A number of regulatory genes that influence dehalogenase expression have been characterised in dehalogenating

organisms. The classical haloacid dehalogenases are usually regulated, which is not surprising because they are natural compounds. In several cases, transcription is mediated by an alternative RNA polymerase containing the σ^{54} factor.

Regulated gene expression is also found for chloroalkane and dichloromethane metabolism, where a negative regulator controls transcription. Surprisingly, even the synthesis of dehalogenases for xenobiotic compounds such as tetrachloroethene and hexachlorocyclohexane (LinD) seems to be regulated [22].

Examples of primitive gene expression can be found in organisms that utilise some xenobiotic haloalkanes. In *Pseudomonas pavonaceae* 170, which utilises 1,3-dichloropropene, *Mycobacterium* sp. strain GP1, which utilises 1,2-dibromoethane, and *X. autotrophicus* GJ10, which utilises 1,2-dichloroethane, expression of the initial dehalogenase is constitutive. The plasmid-localised haloalkane dehalogenase genes (*dhaA*) found in various subspecies of *Rhodococcus erythropolis*, which are assumed to be the source of the dehalogenase genes now also present in *P. pavonaceae* and *Mycobacterium* sp., are regulated by the product of the adjacent *dhaR* gene in the parental *Rhodococci* [2].

The DhaR protein belongs to the TetR family of transcriptional repressor type regulators and responds to 1-chlorobutane and several other 1-halo-*n*-alkanes; however, this protein is not produced in *P. pavonaceae* 170 and is inactivated by a short deletion in the *Mycobacterium* sp. strain GP1 — this deletion was probably a necessary evolutionary step because the repressor cannot be inactivated by the respective substrates.

Acquisition and distribution of dehalogenase genes

Gene transfer is an important process during the evolution of novel catabolic pathways. Acquisition of foreign DNA by horizontal gene transfer requires integration into a replicon that is well maintained in the recipient microorganism. Transposition and gene integration are key mechanisms for the formation of stable new constructs, and different gene integration processes have been suggested. For example, Ravatn *et al.* [20] have proposed that the *clc* element, which encodes the chlorocatechol degradation genes, integrates itself into the *Pseudomonas* B13 chromosome in a site-specific manner by means of the *intB13* gene product, which is encoded by a gene located close to the *clc* genes. Similar site-specific integration processes have been found in phages. In both systems, integration occurs in the 3' end of a target tRNA gene, but due to the identity of the sequences involved in integration (*attB* and *attP*) the original functional tRNA sequence is maintained.

Another way of distributing dehalogenase genes has been the acquisition by a functional replicon of a new dehalogenase gene. This can be mediated by an integrase: both in *P. pavonaceae* 170 and in *Mycobacterium* sp. strain GP1, a gene encoding a putative site-specific recombinase (*intP* in strain

170 and *intM* in strain GP1) is found directly upstream of a haloalkane dehalogenase gene that has probably been recruited from a (Gram-positive) *Rhodococcus* capable of chlorobutane degradation [2].

The *intP* and *intM* gene products share significant sequence similarity with members of the integrase (Int) family of site-specific recombinases. These putative integrase proteins might have mediated insertion of the dehalogenase genes into the bacterial genome. We envisage that such integrase-mediated gene acquisition would occur in a similar manner to the acquisition of antibiotic resistance markers. In the case of these markers, genetic elements (integrons) are found that carry a gene for a site-specific DNA integrase, which can catalyse the incorporation of one or more foreign genes in a site directly upstream of the integrase gene.

Conclusions and future perspectives

There are still several gaps in our understanding of microbial dehalogenation. First, thermodynamic analysis shows that the range of physiological processes that should allow microbial growth at the expense of halogenated substrates is much broader than is found experimentally. This implies that there are biochemical limitations, which might be overcome by further genetic adaptation. This has been the case for aerobic degradation of important organohalogenes, such as 1,2-dichloropropane, 1,2,3-trichloropropane and the dichloroethanes.

Second, little attention has been paid so far to the role of auxiliary enzymes involved in the metabolism of halogenated hydrocarbons. Recently, it has been shown that a functional DNA polymerase I, which is involved in DNA repair, is necessary, in addition to a functional catabolic pathway, for the utilisation of dichloromethane [21]. The requirement for additional detoxification mechanisms may be a more general phenomenon, which would explain the failure of various experiments aimed at the genetic construction of organisms with new catabolic pathways.

Third, unravelling new mechanisms of dehalogenation, such as those of haloalcohol dehalogenases, chloroacrylic acid dehalogenases and hexachlorocyclohexane dehydrochlorinase, remains a challenge. Recently, a mechanism based on elimination of a 1,2-biaxial HCl pair has been proposed for hexachlorocyclohexane dehydrochlorinase, which has been predicted to share similarities with hydrates and isomerases [22,23•].

Fourth, the origin and distribution of dehalogenase genes still present a challenging area of research. In particular, the mutations (including, for example, recombinations) that are associated with the recent adaptation process to anthropogenic pollutants deserve more attention.

Last, recent developments in directed evolution techniques may facilitate the construction of enzymes and organisms with degradation capacities that are not easily obtained by classical adaptation and enrichment.

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